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STUDIES OF THE MECHANISM OF CHLOROQUINE BINDING TO SYNTHETIC DOPA-MELANIN

KRYSTYNA B. STĘPIEŃ and TADEUSZ WILCZOK*
Institute of Chemistry and Physics, Silesian Medical Academy, Sosnowiec, Poland

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Abstract—In order to elucidate the mechanism of drugs binding to melanin, effects of pH, ionic strength and organic solvent on the interaction of chloroquine with synthetic dopa-melanin were studied. The results indicate that electrostatic, hydrophobic and van der Waals' forces participate in the formation of the chloroquine–melanin complex. Binding analysis by the Scatchard method showed that two classes of binding sites take part in the complex formation: strong binding sites with the association constant $K_1 \sim 10^5$ and weak binding sites with $K_2 \sim 10^4$. Experiments with chemically modified melanin yielded some information about binding sites of this biopolymer. The obtained results suggest that strong binding involves both hydrophobic interaction and electrostatic attraction between the protonated ring system of chloroquine and the ortho-semiquinone groups of melanin. However, the weakly reacting sites can be identified as ionic bonds between protonated aliphatic nitrogen of chloroquine molecule and carboxyl groups of melanin. Van der Waals' forces occurring at the conjunctions of the aromatic rings of the drug and the aromatic indole-nuclei of the melanin probably take part in the weak binding too.

The drugs accumulation in pigmented tissues is of considerable interest, not only in pharmacology but also in toxicology. The melanin affinity of the phenothiazines and chloroquine is generally believed to be the most important factor in the etiology of the toxic retinopathy caused by these drugs [1–6]. Hyperpigmentation of the skin, hair bleaching, otic lesions and irreversible extrapyramidal disorders also seem to be due to binding of the drugs to melanin [7–12]. The nature of the interaction between drugs and melanin is still not established. It is assumed to be a charge-transfer process, in which melanin plays the role of the electron acceptor and the bound compounds act as the electron donors [13–15]. However, Larsson *et al.* [16] have shown that the quaternary dipyridylium salts, paraquat and diquat, which are by themselves strong electron acceptors, are bound to melanin *in vivo* and *in vitro*. The results obtained indicated that electrostatic forces may play an important role in the complex formation of some compounds with melanin. Atłasik *et al.* [17] have suggested participation of ionic bonds in the drug–melanin interaction.

The aim of this study was to elucidate the mechanism of drug binding to melanin. We have chosen to investigate chloroquine as it is known for its high affinity for melanin. The data on the effect of pH, ionic strength and organic solvents on the interaction of this drug with melanin are presented. In order to obtain some information on binding sites of this biopolymers the binding of chloroquine to methylated melanin was also investigated.

MATERIALS AND METHODS

Materials. Melanin was synthesized *in vitro* by autooxidation of (±)-3,4-dihydroxyphenylalanine (dopa) at pH 8.0 according to the method described by Binns *et al.* [18]. Methylated melanins were obtained by treatment of dopa-melanin with excess of diazomethane and with methanol saturated by gaseous hydrogen chloride, according to the procedures described by Swan and Waggott [19].

The mean size of the melanin granules used in our experiments was 0.61 μm^2 as determined by total surface projection measurements. The standard deviation was 0.47. About 300 granules were measured for size distribution. The histogram shows that about 80–85% of the granules belongs to the 0.5–0.7 μm^2 class but the extremes were 0.26 and 2.02, respectively. The computing scanning microscope MORPHOQUANT was used for evaluation of the size distribution parameters.

Chloroquine diphosphate was obtained from Polfa Pabianice, Poland. The drug was dissolved in the buffer solutions at pH over the range 8.0–2.2. Spectrophotometric measurements were made using the low range u.v. Cary 118 C spectrophotometer. The molar extinction coefficients of chloroquine were determined at 343 nm for each solution.

Binding of chloroquine to melanin. Binding of chloroquine to melanin was studied in 0.067 M phosphate buffer at pH 7.0, except experiments in which the effect of pH was investigated.

Five milligrams of melanin were placed in Erlenmeyer flasks. The buffer and chloroquine solution were added to each flask to final volume of 10 ml. The initial concentration of the drug ranged from 1×10^{-5} to 1×10^{-3} M. Control samples contained 5 mg of melanin and 10 ml of buffer. Samples were kept at room temperature for 20 hr. This incubation time was found in preliminary experiments described previously by Atłasik *et al.* [17]. Such suspensions were filtered and the absorbance of each filtrate with respect to the control sample was measured. The concentration of chloroquine remaining in each fil-

* Address for correspondence: Prof. Dr. hab. Tadeusz Wilczok, Institute of Chemistry and Physics, Silesian Medical Academy, 41200 Sosnowiec, ul. Jagiellońska 4, Poland.

trate and the amount of the drug bound to melanin was then determined.

In some experiments, sodium chloride (0.4–2 M) was added to the examined solutions.

For the investigation of organic solvents influence on melanin-binding properties, 1, 2 and 5 ml of methanol, ethanol and *n*-propanol were added to the incubation medium.

Recovery of chloroquine from melanin–drug complexes. Chloroquine was complexed with melanin as described above. The melanin granules were separated by centrifugation and then successively washed with 10 ml portions of 0.067 M phosphate buffer, pH 7.0, 4 M sodium chloride and 95% ethanol. After each washing the melanin was centrifuged and the absorbance value of each supernatant was measured to determine the amount of eluted chloroquine. The washings were repeated until the absorbance of the supernatants was nearly zero.

Analysis of chloroquine binding. The binding of chloroquine to melanin was analysed by the modified Scatchards method [21, 22]. Assuming that chloroquine belongs to drugs demonstrating binding to several classes of binding sites with the constant K_i for each of the n_i sites in class i, it is possible to calculate the amount of the drug bound per milligram of melanin r, based on c representing the concentration of free drug, according to the equation:

$$r = \sum_i r_i = \sum \frac{n_n K_i c}{1 + K_i c}$$

If there was only one class of binding sites, a plot of r/c versus r is a straight line with an intercept on the r axis equal to n and with a slope of $-K$. The presence of more than one class of binding sites results in a curved r/c versus r plot. When two or three types of binding sites are present, it is possible to discern linear portions in the plot of r/c versus r from which the individual n_i and K_i can be determined.

It is known that the procedure of drawing tangents to the apparently linear portions of the curve does not give accurate measures of the true values. Therefore, these values were not taken for direct binding constants determination. From the experimentally constructed curve where r/c is presented as a function of f/r the following equations can be drawn:

$$a = n_1 + n_2 \; (r \text{ axis when } c \to \infty)$$

$$b = n_1 \cdot K_1 + n_2 \cdot K_2 \; (r/c \text{ axis when } c \to 0)$$

$$c = -\frac{n_1 K_1^2 + n_2 K_2^2}{n_1 K_1 + n_2 K_2} \; (\text{slope when } c \to 0)$$

$$d = -\frac{n_1 + n_2}{\frac{n_1}{K_1}} + \frac{N_2}{K_2} \; (\text{slope when } c \to \infty)$$

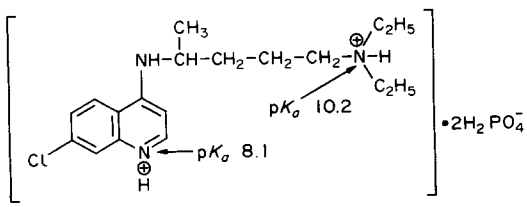

Fig. 1. Structural formula of chloroquine diphosphate.

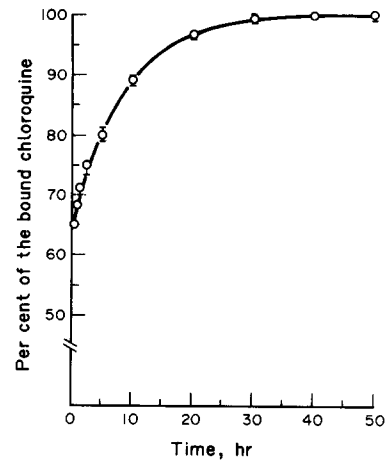

Fig. 2. Effect of incubation time on chloroquine bound to melanin. Ten ml of 10^{-3} M chloroquine solution, K/Na phosphate buffer at pH 7.0, incubated with 5 mg of melanin.

From this data n_1, n_2, K_1 and K_2 were determined manually. Such solving of the four equations was proposed by H. Schütz (personal communication) and checked for other ligand binding to biopolymers with good results.

RESULTS

Binding of chloroquine to melanin. The adsorption of chloroquine to synthetic dopa-melanin as a function of incubation time is shown in Fig. 2. It can be seen that the amount of bound chloroquine shows an increase during the time course of the experiment up to 35 hr of incubation. When the adsorbed amount of the drug after 35 hr was used as 100%, it was found that 65% of chloroquine was adsorbed on melanin in the first 30 min and about 98% after 20 hr.

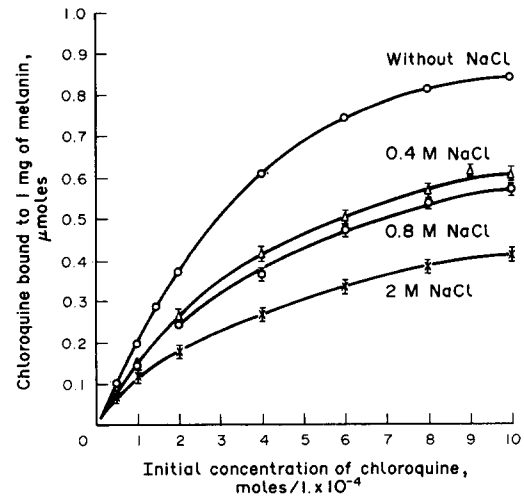

Fig. 3. The binding of chloroquine to melanin at various NaCl concentrations. Variable amounts of chloroquine were incubated with 5 mg of melanin in K/Na phosphate buffer at pH 7.0 containing given amounts of NaCl.

Binding of chloroquine to dopa-melanin studied in phosphate buffer, pH 7.0, is shown in Fig. 3. The number of μmoles of chloroquine bound to 1 mg of melanin is plotted as a function of the initial concentration of the drug. It can be seen that the binding is concentration dependent. At low initial concentrations of the drug the percentage of chloroquine bound to melanin is high. When the concentration increases the absolute amount of fixed chloroquine is higher, but the relative amount decreases. This may indicate that chloroquine is bound to melanin up to complete saturation of all binding sites present in the biopolymer. The detailed analysis of the ionic strength effect was omitted because it was evidently shown by Larsson and Tjälve [23] that concentrations of Na^+ and K^+ in buffered media used in their experiments were too low to exert any marked interference in binding of drugs to melanin.

The concentration of the buffer solution in our experiments was identical as in the Larsson and Tjälve experiments and therefore it is difficult to expect any effective influence from the cationic contribution of the buffer on the binding of chloroquine to melanin.

The results obtained in our experiments were analysed by Scatchard's method which can provide information concerning the number and nature of the binding sites. The plot *r/c* versus *r* for the binding of chloroquine to dopa-melanin is presented in Fig. 4. It was assumed that the observed nonlinearity of this plot resulted from heterogeneity of binding sites rather than from electrostatic factors since even high ionic strength did not eliminate curvature. The form of *r/c* versus *r* plot indicates that two classes of binding sites must be implicated in the interaction of chloroquine with melanin: strong binding sites with the association constant $K_1 = 3.91 \times 10^5$ M^{-1}, and weak binding sites with $K_2 = 1.07 \times 10^4$ M^{-1}. The number of strongly reacting sites (n_1) and weakly reacting sites (n_2) are given in Table 1. The total binding capacity (n_1) of dopa-melanin for chloroquine is 0.94 μmoles/mg.

It was shown in preliminary experiments that concentrations higher than 10^{-3} M/l of chloroquine do not change the adsorption rate on melanin. This was valid also for melanin preparations obtained from cattle eyes. Therefore, the presence of additional binding classes in this concentration range does not seem possible.

Recovery of chloroquine from melanin–drug complex. The melanin–chloroquine complex was washed several times with various solutions in order to explain the nature of the interaction between melanin and the drug. The elution profile for melanin–chloroquine complex is presented in Fig. 5. As one can see, the binding of chloroquine to melanin is reversible. Repeated washings of the complex removed about 97% of bound chloroquine. It is apparent from the data in Fig. 5 that the highest amount (about 53%) of the bound drug is eluted with 0.067 M phosphate buffer. The amount of chloroquine washed out with 4 M NaCl and ethanol is 21 and 23%, respectively.

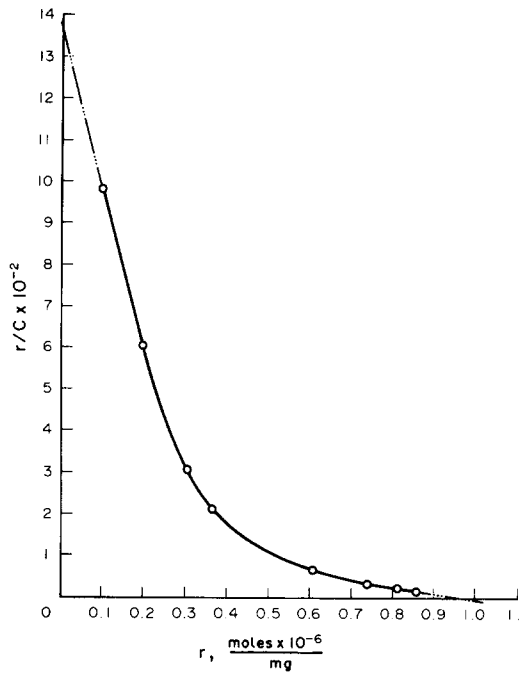

Fig. 4. Scatchard plot for the melanin-binding of chloroquine. *r*, μmoles of chloroquine bound per mg melanin; *c*, concentration (M) of the free drug.

Effect of ionic strength. The effect of ionic strength on the interaction of chloroquine with melanin is shown in Fig. 3. It can be seen that increase in salt concentration diminishes the amount of bound chloroquine.

The data obtained in these experiments yielded a nonlinear plot of *r/c* versus *r*, suggesting the interpretation of binding in terms of two classes of sites. The analysis of the data yielded in binding parameters are presented in Table 1. A comparison of these values indicate that increase in ionic strength decreases the number of strongly and weakly reacting

Table 1. Binding parameters for the interaction of chloroquine with melanin at various salt concentrations at pH 7.0

NaCl concn (mole/l)	K_1	K_2	n_1	n_2
0.0	3.91×10^5	1.07×10^4	0.32	0.62
0.4	1.77×10^5	0.33×10^4	0.18	0.62
0.8	1.74×10^5	0.33×10^4	0.14	0.59
2.0	0.89×10^5	0.19×10^4	0.13	0.52

K_1, K_2 (M^{-1}), association constants for strongly and weakly reacting binding sites.
n_1, n_2 (μmoles per mg melanin), number of binding sites.

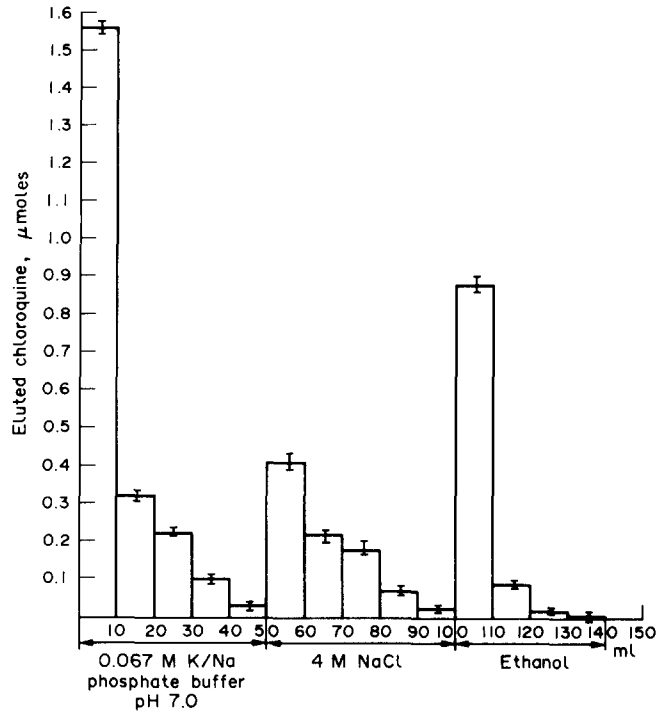

Fig. 5. Recovery of bound chloroquine after washings with phosphate buffer, sodium chloride and ethanol.

sites and reduces the association constant values for both types of binding sites.

Effect of pH. The effect of pH on the interaction between chloroquine and melanin was studied within a range of pH 2.2–8.0. It was found that the absorption spectra of chloroquine have a double peak in the 325–345 nm region within the pH range examined. This characteristic double peak of chloroquine represents the doubly protonated species, whereas the single band near 330 nm represents the species protonated only at the side chain nitrogen atom [24]. The effect of pH on the interaction of chloroquine with melanin is shown in Fig. 6. It can be seen that with the lowering of pH, the amount of bound chloroquine decreases remarkably.

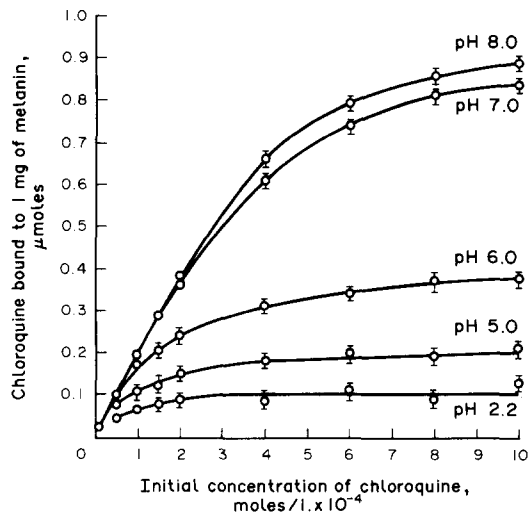

Fig. 6. Effect of pH on binding of chloroquine to melanin. The pH values over the range 8.0–5.0 were obtained using 0.067 M phosphate buffer (according to Sorensen); for pH 2.2, 0.2 M disodium hydrogen phosphate and 0.1 M citric acid were used.

Scatchard analysis of the data obtained in these experiments showed that two bindings classes participate in the interaction of chloroquine with melanin at pH over the range 8.0–5.0. The lowering of pH caused decrease of the association constant values and the number of strongly and weakly reacting binding sites (Table 2). The plot r/c versus r for the data obtained at pH 2.2 yielded a straight line which demonstrated that only one set of binding sites participate in the interaction of the drug with melanin. In this case n and K values may be brought into a relationship with those determined for the weakly reactive binding sites.

Table 2. Binding parameters for the interaction of chloroquine with melanin at various pH values

pH values	K_1	K_2	n_1	n_2
8.0	4.21×10^5	0.84×10^4	0.40	0.58
7.0	3.91×10^5	1.07×10^4	0.32	0.62
6.0	1.59×10^5	0.29×10^4	0.24	0.25
5.0	1.33×10^5	0.28×10^4	0.12	0.12
2.2*		1.89×10^4		0.11

K_1, K_2 (M^{-1}), association constants for strongly and weakly reacting binding sites.

n_1, n_2 (μmoles of chloroquine per mg melanin), number of binding sites.

* At pH 2.2 no strong binding of chloroquine to melanin was shown.

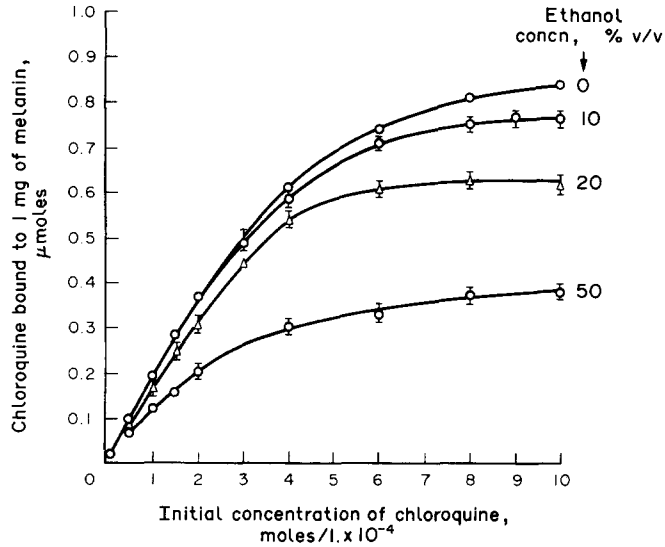

Fig. 7. The binding of chloroquine to melanin in the presence of various amounts of ethanol.

Effect of organic solvents. The effect of methanol, ethanol and *n*-propanol on the interaction of chloroquine with melanin was investigated. The volume percentages of the alcohols varied from 0 to 50. Figure 7 represents chloroquine binding in the presence of increasing amounts of ethanol. It can be seen that the addition of ethanol apparently decreases the binding of chloroquine to melanin. Similar effects were also found for methanol and *n*-propanol.

The influence of the same amount of the tested alcohols (50%) on the interaction between chloroquine and melanin was also investigated. It was found that the effectiveness decreased in the order: propanol > ethanol > methanol.

Scatchard analysis of the data obtained in these experiments yielded binding parameters which are presented in Table 3. In the presence of a small amount of alcohol (10%), two binding classes participate in the interaction of chloroquine with melanin. When the amount of alcohol increases one class of the binding sites decay. A comparison of the association constant values demonstrates that in this case only weakly reacting sites participate in the binding of chloroquine to melanin.

Interaction of chloroquine with methylated melanins. In order to obtain some information on the participation of carboxy- and hydroxy-groups of melanin in the drug binding, the interaction of chloroquine with methylated melanins was studied. Methylated melanins were obtained by the action of methanolic hydrogen chloride or diazomethane. Treatment of melanin with methanol in the presence of hydrogen chloride results in the methylation of carboxy-groups, whereas the treatment with excess of diazomethane involves methylation of carboxy- and hydroxy-groups as well as indole- and pyrrole NH-groups [19].

The binding of chloroquine to the ester of melanin is presented in Fig. 8. It can be seen that the blocking of carboxy-groups causes a decrease of the amount of chloroquine bound to melanin. Interaction of chloroquine with esterified melanin resulted in a curved plot of r/c versus r. Analysis of the data yielded values of $n_1 = 0.31$, $n_2 = 0.33$, $K_1 = 6.4 \times 10^5$ and $K_2 = 0.13 \times 10^4$.

Table 3. Effect of ethanol on the binding parameters of chloroquine with melanin

Per cent of ethanol	K_1	K_2	n_1	n_2
10	2.83×10^5	1.38×10^4	0.28	0.56
20	*	2.2×10^4		0.71
50		0.97×10^4		0.43

K_1, K_2 (M^{-1}), association constants for strongly and weakly reacting binding sites.

n_1, n_2 (μmoles of chloroquine per mg melanin), number of binding sites.

* In the presence of 20 and 50% (v/v) of ethanol no strong binding of chloroquine to melanin was shown.

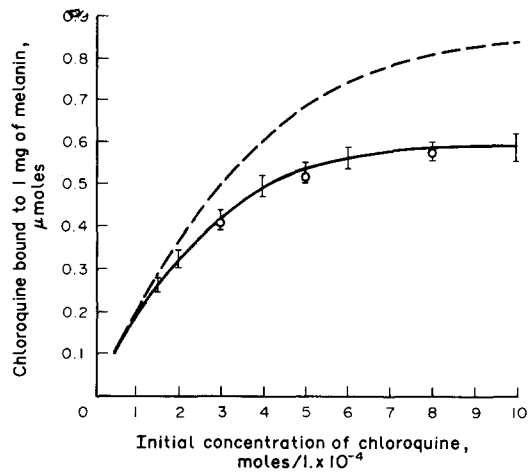

Fig. 8. The binding of chloroquine to the methyl ester of melanin. Dotted line represents binding of chloroquine to unmethylated melanin.

Binding sites of chloroquine to melanin treated with diazomethane demonstrated that chloroquine has not revealed any significant affinity to this melanin.

DISCUSSION

The presented investigations show that the interaction of chloroquine with synthetic dopa-melanin can be described in terms of two distinct classes of binding sites: class I comprises strongly reacting sites ($K \sim 10^5$) and class II comprises weakly reacting sites ($K \sim 10^4$). Shimada *et al.* [25] have characterized the drug binding process in terms of one type of binding site, but these investigators used a low initial concentration of the drug. On the other hand, Larsson and Tjälve [23] have found that the interaction of chloroquine with melanin can be best described by assuming three classes of binding sites. However, these authors have employed in their studies pigments from bovine eyes, and it cannot be excluded that the protein moiety takes part in the binding process to the pigment granules. One has to take into consideration that because of difficulties in deproteinisation of isolated melanins as well as in their purification from trace elements, the analysed melanin samples originating from pigmented tissues may offer incomparable drug binding possibilities as compared to synthetic melanins synthesized from chemically pure reagents dissolved in deionized water. Therefore, it may be that the three classes of binding sites shown by Larsson and Tjälve are related to some specific melanin preparations only. The question that the biphasic Scatchard plots might represent heterogeneity of our melanin preparation rather than two types of binding sites on a single homogeneous class of molecules has also to be analysed. It is evident from our data that the melanin samples used are not homogeneous in size and shape. It is also impossible to say something about the chemical construction of each single melanin granule. On the other hand, we have shown that the ratio of strong to weak binding sites in the analysed melanin granules was 1:2. Our histograms of melanin size and shape distribution did not reflect a situation where two classes of melanin granules are present in a ratio of 1:2. Thus, it is difficult to explain the presence of two binding sites based on heterogeneity in size and shape only. Recovery of chloroquine from the melanin–drug complex proves that this combination is reversible. The use of various solutions for elution of chloroquine from the melanin–chloroquine complex makes possible the explanation of the nature of interaction between melanin and the drug. The elution of chloroquine by the use of neutral buffer solution may point to a participation of van der Waals' forces occurring at the conjunctions of the aromatic rings, of the drug and the aromatic indole-nuclei of the melanin in the melanin–drug complex. Recovery of chloroquine by means of high ionic strength solution indicates that electrostatic forces can play an important role in the complex formation. The elution of the drug with ethanol suggests that the hydrophobic interactions cannot be ruled out.

Generally, as it was shown by Bridelli *et al.* [26], by the use of infrared band analysis of synthetic and natural melanins, these polymers have a polyelectrolyte character and therefore their ability to form complexes with chloroquine can be explained at least partially by direct ionic attraction between the positively charged chloroquine molecules and anionic sites of melanin. Examination of the effect of pH and ionic strength on chloroquine binding and analysis of the obtained data indicate that electrostatic forces take part in both the strong binding and the weak binding.

In order to explain the role of carboxyl and hydroxyl groups, the binding of chloroquine to methylated melanins was studied. Melanin contains free carboxylic acid groups in addition to phenolic and quinonoid groups [19, 27, 28] and these groups are presumably responsible for the complex formation. It was found that the methyl ester of melanin has smaller ability for chloroquine binding than non-methylated melanin.

A comparison of binding parameters for the interaction of chloroquine with melanin ester and unmodified melanin indicates that the blocking of carboxyl groups decreases the number of weakly reacting sites; however it does not alter the number of strongly reacting sites. Therefore, the ionic interaction between chloroquine and carboxyl groups of melanin represents the weak binding.

Melanin treated with diazomethane has not demonstrated any affinity to chloroquine. It indicates that exhaustive methylation causes not only the blocking of anionic sites on melanin but also presumably precludes the electron transfer processes between indole units. Data collected by Thathachari [29–31] obtained by the use of X-ray diffraction methods demonstrated that indole or other aromatic units present in melanin are stacked upon one another to form a π-complex. It cannot be excluded that exhaustive methylation produces sterical hindrances for the interaction of chloroquine with melanin.

Additional information on the nature of the interaction of melanin with chloroquine was obtained when investigating the stability of the complexes in the presence of methanol, ethanol and *n*-propanol. All organic solvents used decrease the binding ability of chloroquine. The effectiveness of the solvents increases with their hydrocarbon content and the complex formation is effectively suppressed by organic solvent concentration. These results appear to demonstrate the importance of hydrophobic forces for the interaction of chloroquine with melanin in aqueous solution. It is possible too, that the reduced binding of chloroquine to melanin may be due to alteration of the drug properties induced by the organic solvents used. When the absorption spectra of chloroquine were measured in various alcohol–water mixtures, it was found that in the presence of increasing amounts of alcohol the spectrum of chloroquine gradually changes from one having a double peak in the 325–345 nm region to a single peak spectrum at 330 nm. These spectral changes resulted from a progressive decrease in protonation of the ring nitrogen atom of the chloroquine molecule [24]. It is possible that the disappearance of strongly reacting sites in the presence of alcohol resulted from

lack in protonation of the quinoline ring. Consequently, if chloroquine exists in the doubly protonated form then the drug is strongly bound to melanin. This conclusion resulted also from the decreased number of strongly reacting sites with increasing ionic strength and lowering pH. Presumably the strong ionic bond involves *ortho*-semiquinone groups of stacked indole units of melanin. Felix *et al.* [32] demonstrated that *ortho*-semiquinone free radicals centres in melanin have the ability to bind cations forming chelate complexes. The weak ionic bond may be formed between the aliphatic nitrogen atom of chloroquine and the carboxyl group of melanin.

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